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The Effect of Monovalent Ions on Polyphosphate Binding to *Escherichia coli* Exopolyphosphatase[1]

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The thermodynamic driving force for the interaction of *Escherichia coli* exopolyphosphatase (PPX) with polyphosphate was investigated by varying salt choice and concentration. This interaction was found to be cation concentration independent but weakly dependent on the concentration of certain anions. Both of these traits are very uncommon for nonspecific protein–polyelectrolyte interactions. Interpretation of these results based on theory indicated that binding was not entropy driven due to release of polyelectrolyte-condensed counterions, as is the case for nearly all protein–polyelectrolyte interactions. The thermodynamics of the PPX–polyphosphate interaction showed similarity only to the interaction of polynucleotides with single stranded binding proteins.

Key Words: **anion; cation; equilibrium binding; dissociation constant; polyphosphate; PPX.**

Escherichia coli exopolyphosphatase is a processive enzyme that hydrolyzes terminal phosphates from polyphosphate. The enzyme is composed of two independent structural domains with the N-terminal domain having weak sequence similarity to the active site region of the sugar kinase/actin/hsp70 superfamily (1). An active site was confirmed to exist on the N-terminal domain along with two weak polyphosphate binding sites (2). Although only a single polyphosphate binding site was identified on the C-terminal domain, this site bound polyphosphate as tightly as native PPX and this C-terminal domain was required for the highly processive mode of action of the enzyme (2). The thermodynamic driving force of polyphosphate binding by PPX has not previously been addressed.

Extensive characterization of the thermodynamics of protein–polyelectrolyte interactions, however, has occurred including characterization of proteins interacting with the phosphate backbone of DNA and RNA (3, 4). Many of these interactions are electrostatic with binding driven by entropy from release of polyelectrolyte bound cations (5, 6). Characterization of these interactions has often involved the effect of salt choice and concentration on the strength of the protein–polyelectrolyte interaction.

This report describes the development of an assay for measuring polyphosphate binding by PPX. The assay was used to characterize the effect of salt concentration on polyphosphate binding for various salts. The data was compared with that of other protein–polyelectrolyte interactions and was analyzed using Manning's counterion condensation theory (7, 8) and the protein–polyelectrolyte theory of Record and Lohman (3, 9) to gain an understanding of the thermodynamics of polyphosphate binding by PPX.

MATERIALS AND METHODS

Generation of N- and C-terminal domains of PPX. N- and C-terminal domains of PPX corresponded to limited proteolysis fragments generated using *Staphylococcus* V8 protease. These domains were overexpressed separately and were purified as previously described (2).

Inhibition of PPX activity. PPX was stored in 50 mM Tris–Cl (pH 7.5), 150 mM KCl, and 1 mM $MgCl_2$. Dialysis of this PPX against a 1000-fold volume excess of 50 mM Tris–Cl (pH 7.5), 150 mM KCl, and 2 mM EDTA was used for removal of the Mg^{2+}. To test for recovery of activity using other divalent cations, this dialyzed PPX was diluted 10-fold into reaction mixtures containing specified divalent cations in the range of 1 to 10 mM. Enzyme activity was measured using a standard exopolyphosphatase activity assay involving thin layer chromatography to resolve hydrolyzed from unhydrolyzed $[^{32}P]$-polyphosphate (10).

Phosphate analog AlF_4^- was generated from 50 mM NaF and $AlCl_3$ at the desired Al^{3+} concentration. Phosphate analog VO_4^{3-} was added to reaction mixtures as Na_3VO_4. In both cases reaction buffers contained 50 mM Tris–Cl (pH 7.5), 150 mM KCl, and 1 mM $MgCl_2$ supplemented with the appropriate phosphate analog.

Potential terminal phosphate chelator $Cr(tacn)(CF_3SO_3)_3$ ("Cr-tacn") was generated from $Cr(tacn)(CO)_3$ (11) through the intermediate $Cr(tacn)Cl_3$ (12). A second potential terminal phosphate chelator

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$[Cr(tren)(CF_3SO_3)_2](CF_3SO_3)$ ("Cr-tren") was generated from $[Cr(tren)Cl_2]Cl$ (13, 14). The PPX-polyphosphate reaction contained 50 mM Tris–Cl (pH 7.5), 150 mM KCl, 1 mM $MgCl_2$, 69 μM polyphosphate chains, 7 nM PPX (dimers), and either 138 μM (1×) or 690 μM (5×) chelator.

Immobilization of PPX. PPX was immobilized using *N*-hydroxysuccinimide (NHS) activated acrylic beads (Affiprep 10, Bio-Rad). The coupling reaction occurred with nucleophilic attack by nitrogen lone pair electrons displacing the NHS group. Lysine is the most likely primary amine nucleophile though other residues may also participate.

NHS-acrylic beads were washed with 50 volumes of ice-cold 10 mM sodium acetate (pH 4.5) followed by 20 volumes of 50 mM HEPES (pH 7.0) with 175 mM KCl. The gel was resuspended in one-half volume of this HEPES-KCl buffer. Coupling reactions (1.75 ml) contained 150 μl PPX (approximately 12 mg PPX/ml in 50 mM HEPES (pH 7.0) with 175 mM KCl), 850 μl HEPES-KCl buffer, and 750 μl resuspended gel. Reactions took place with slow mixing at 4°C for 3 h. Unreacted gel sites were blocked by the addition of 100 μl of 1 M ethanolamine (pH 8.0) with the reaction continued at 4°C for an additional 15 min. The gel was washed with 3 volumes of buffer containing 50 mM HEPES (pH 7.5) and 0.5 M KCl followed by 50 volumes of 10 mM HEPES (pH 7.5) with 175 mM KCl and 0.5 mM EDTA. This final buffer was also used to resuspend the gel (500 μl buffer per 1.75-ml reaction). Purified N- and C-terminal domains were also immobilized with this method.

Immobilized enzyme equilibrium binding assay. Reaction volumes of 10 μl contained 4 μl resuspended, immobilized PPX and 6 μl of a 10 mM HEPES (pH 7.5), 0.5 mM EDTA buffer. This 6-μl solution was also supplemented with polyphosphate and the desired salt to give an equilibration buffer with the correct final polyphosphate and salt concentrations assuming 8 μl of final liquid reaction volume and 2 μl of solid, immobilized enzyme. After a short equilibration period of approximately 10 s the reaction was mixed with 100 μl of equilibration buffer without polyphosphate. This was transferred to an ST 69 cellulose acetate membrane (Schleicher and Schuell, Keene, NH) on a vacuum filtration apparatus. The gel was then washed with 10 ml of equilibration buffer without polyphosphate and PPX bound polyphosphate was measured by liquid scintillation counting of the membrane. Levels of polyphosphate retained during filtering were constant with rinse volumes greater than 5 ml and remained constant even after rinsing with 50 ml of wash buffer.

Equilibrium association constants (K_a) were determined from Scatchard plots containing data at a minimum of four polyphosphate concentrations. Polyphosphate binding was measured at least twice for each polyphosphate concentration. Scatchard plots were not linear but data was split to determine effective association constants at "high" and "low" polyphosphate concentrations using best-fit lines. As higher polyphosphate concentrations may give rise to nonspecific binding only the "low" polyphosphate concentration K_a values were determined. These K_a values were determined from the slopes of the Scatchard plot best-fit lines using preset y-intercepts. These intercepts were determined for each salt by averaging the true best-fit-line y-intercepts at each concentration of a given salt.

RESULTS

Inhibition of PPX Activity

A method of inhibiting PPX activity was sought to allow investigation of substrate binding without polyphosphate hydrolysis.

As many phosphatases require divalent cations for activity, this requirement for PPX, as well as the activity of PPX with group IIA series divalent cations, was measured. As a percentage of maximum activity group IIA series cations had 100% activity for Mg^{2+}, 12% for Ca^{2+}, 0.3% for Sr^{2+}, and 2% for Ba^{2+}. Removal of divalent cations by dialysis with EDTA caused activity to fall to 0.3% of maximum. This removal of divalent cations was used during the investigation polyphosphate binding.

TABLE 1

Inhibition of PPX Activity with Cr-tacn and Cr-tren Reported as Percent of Uninhibited Activity (V_{max})

Concentration relative to [polyphosphate]	Cr-tren	Cr-tacn
1×	80%	22%
5×	37%	6%

Additional methods of inhibiting activity were also characterized. For example, substrate analogs AlF_4^- and VO_4^{3-}, inhibitors of many phosphatases (15), were tested for their ability to inhibit PPX activity but were found to be poor inhibitors. At 1 mM VO_4^{3-} PPX activity was reduced by less than 5% while 10 mM AlF_4^- reduced activity by only 25%.

Also, carboxylic acid-chelating Cr-tacn and Cr-tren (14, 16) were tested for their ability to inhibit PPX activity by chelating the carboxyl-like, terminal phosphates of polyphosphate. While Cr-tren was able to significantly lower PPX activity, Cr-tacn was a much more effective inhibitor (Table 1). The mechanism of this inhibition was not probed to confirm chelation of terminal phosphates rather than carboxyl groups of PPX.

Effects of Salt Concentration on the PPX-Polyphosphate Interaction

An immobilized enzyme equilibrium binding assay was used to determine the effect of salt concentration on polyphosphate binding by PPX for various salts.

The effect of salt concentration on K_a for potassium salts was found to be dependent on the choice of anion (Fig. 1). Values of $\partial \log K_a/\partial \log[\text{salt}]$ range from -0.15 for $KOOCCH_3$ to -2.8 for KSCN (Table 2). Changing cations had little effect on $\partial \log K_a/\partial \log[\text{salt}]$. For example, $\partial \log K_a/\partial \log[\text{salt}]$ was -0.15 for $KOOCCH_3$ versus -0.12 for $NaOOCCH_3$ while KCl_2 and NH_4Cl both had a value of -1.1.

The immobilized enzyme binding assay was also used to measure the effect of salt concentration on polyphosphate binding to purified C-terminal domain. This C-terminal domain and PPX were found to have very similar anion choice and salt concentration effects (Fig. 2).

DISCUSSION

This paper reports both the development of a method for measuring enzyme-polyphosphate binding and the

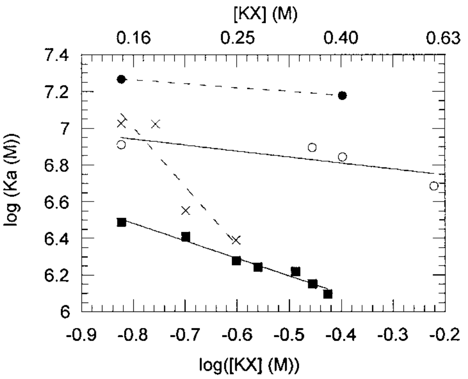

FIG. 1. Effect of concentration for various potassium salts on the equilibrium association constant of polyphosphate binding to PPX. Data points represent KCl (■), KOOCCH$_3$ (○), KF (●), and KSCN (×).

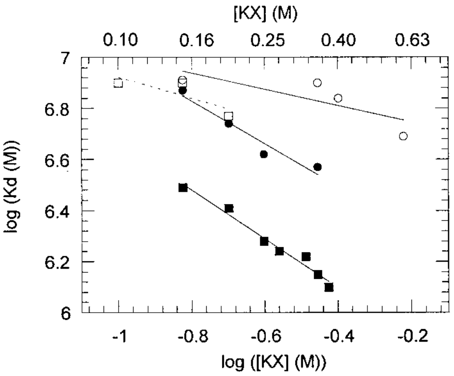

FIG. 2. Effect of KCl and KOOCCH3 concentrations on the equilibrium association constant of polyphosphate binding to PPX or to purified C-terminal domain. Data points for polyphosphate binding to PPX are KCl (■), and KOOCCH$_3$ (○), and for binding to purified C-terminal domain are KCl (●), and KOOCCH$_3$ (□).

use of this method in investigating the thermodynamics of the PPX-polyphosphate interaction.

Inhibition of PPX Activity

Requirement of divalent cations is a common feature for proteins in the sugar kinase/actin/hsp70 superfamily. For proteins of this superfamily with known structures, these divalent cations are found in the active site and interact with active-site-bound phosphates (17–19). Consistently, PPX requires Mg^{2+} for optimal activity and has only 0.3% residual activity in the absence of divalent cations. Removal of divalent cations was chosen for investigating equilibrium binding based on convenience and as control studies at low temperatures indicated their presence had a relatively small effect on polyphosphate binding. Unfortunately, in the absence of divalent cations PPX precipitated when mixed with polyphosphate making it difficult to use standard solution-phase techniques for measuring equilibrium binding. To circumvent this precipitation, polyphosphate binding was measured as substrate retained by immobilized PPX or immobilized domains of PPX.

Salt Concentration Effects on Polyphosphate Binding

The effects of salt concentration on protein–polyelectrolyte interactions have been investigated for several decades. This has included extensive characterization of salt-concentration effects on proteins binding to DNA and RNA (3, 6, 9, 20). These interactions are highly salt concentration dependent and data plotted as log K_a versus log[salt] is linear over a concentration range of several hundred mN, with most slopes in the range of -8 to -20 (3). Binding of *E. coli* lac repressor to nonoperator DNA, for example, has a $\partial \log K_a/\partial \log[\text{salt}]$ value of -12, while *E. coli* RNA polymerase binding to ssDNA exceeds the normal range with a $\partial \log K_a/\partial \log[\text{salt}]$ of -25 (3).

TABLE 2

Effect of Concentration for Various Potassium Salts on the Equilibrium Association Constant of Polyphosphate Binding to PPX Indicated by Values of $\partial \log K_a/\partial \log[\text{salt}]$

$\partial \log K_a/\partial \log[\text{salt}]$			
KCl	KF	KSCN	KOOCCH$_3$
-1.1	-0.20	-2.8	-0.15

Values of $\partial \log K_a/\partial \log[\text{salt}]$ for the PPX-polyphosphate interaction from -2.8 to only -0.15 are significantly lower than observed for other protein–polyelectrolyte interactions. The insensitivity of the PPX-polyphosphate interaction to acetate and fluoride salts suggests the interaction is independent of cation concentration while, the KSCN and KCl data show the interaction to be only weakly dependent on the concentration of certain anions.

Effects of anion choice on $\partial \log K_a/\partial \log[\text{salt}]$ are very uncommon for most protein–polyelectrolyte interactions; however, they are clearly observed for PPX. The order of anion effects on $-\partial \log K_a/\partial \log[\text{salt}]$ is $F^- \approx CH_3COO^- < Cl^- < SCN^-$. This sequence of anions is in accordance with the Hofmeister or lyotropic series which sorts ions based on the strength of protein-ion versus water-ion interactions (21, 22). Anions such as SCN$^-$ have stronger protein-ion interactions while those like F$^-$ and acetate have stronger water-ion interactions (3, 22). The accordance of the anion choice dependence with the Hofmeister series suggests these anions bind directly to PPX. While these anions could bind anywhere on the enzyme and have an indirect effect on polyphosphate binding sites, it seems more likely that the anions compete with polyphosphate for a positively charged region of the protein.

Protein–Polyelectrolyte Theory

Several theoretical approaches have been developed for protein–polyelectrolyte interactions. One of these approaches, developed by Lohman, Record, and coworkers (3, 9) has been used extensively for describing the effect of salt on protein–polyelectrolyte interactions (3, 9). This theory makes use of Manning's limiting law counterion condensation theory (7, 8) which predicts monovalent ions interact in two ways with polyelectrolytes. The first is direct condensation of counterions to reduce the contour charge density of the polyelectrolyte. The second is a Debye-Hückel screening of the remaining charge density by co- and counter-ions. Coulombic interaction between protein and charged polyelectrolyte residues will change polyelectrolyte charge density near the region of binding and this change will release condensed counterions. It is expected that the entropy change for this counterion release will depend on the bulk counterion concentration. While the driving force for protein–polyelectrolyte binding is not known *a priori*, the magnitude of this entropy change necessarily affects equilibrium protein–polyelectrolyte binding.

This macroscopic protein–polyelectrolyte theory uses the reaction equilibrium

$$\mathrm{E} + \mathrm{S} \overset{K_a}{\longleftrightarrow} \mathrm{ES} + n_m\mathrm{M} + n_x\mathrm{X} + n_w\mathrm{W}, \qquad [1]$$

where E and S are free enzyme and substrate, ES is the enzyme-substrate complex, M and X are cation and anion, and W is water. The coefficients n_m, n_x, and n_w represent the net release of bound cation, anion, and water upon formation of the ES complex with K_a as the equilibrium constant. Based on Eq. [1] the dependence of K_a on mean ionic activity, $a_\pm$, is given by Eq. [2] (3, 9, 23).

$$\frac{\partial \log K_a}{\partial \log a_\pm} = -\Delta\left(n_m + n_x - \frac{p_m}{55.6}\, n_w\right) + \frac{\partial \log \gamma^0_E \gamma^0_S \gamma^0_{ES}}{\partial \log a_\pm}, \qquad [2]$$

where the Δ terms represent the net change in bound cation, anion and water, m is the molal concentration of electrolyte, p is the aggregate valency of the electrolyte MX, and the γ terms are the activity coefficients for the reference states of E, S, and ES. From Eq. [2], it is observed that a number of factors can effect $\partial \log K_a / \partial \log a_\pm$ including differential cation binding, differential anion binding, differential hydration, and changes in the activity coefficient ratio for E, S, and ES with changes in $a_\pm$.

In nearly all cases, experimental data plotted as log K_a versus log[salt] is linear and insensitive to anion choice (3). Therefore, all terms are dropped with the exception of that for cation release. Over the experimental salt concentration ranges studied, roughly several hundred mN, it is also assumed that replacing the mean ionic activity with salt concentration introduces little error. Equation [2] becomes

$$\frac{\partial \log K_a}{\partial \log[\mathrm{salt}]} = -\Delta(n_m). \qquad [3]$$

Using condensation theory, the number of charge–charge interactions (m') may be estimated with Eq. [3] and the extent of condensation (Ψ) as

$$\Delta(n_m) = -\frac{\partial \log K_a}{\partial \log[\mathrm{salt}]} = m'\Psi. \qquad [4]$$

The extent of condensation takes into account both condensed counterions and the uncondensed Debye–Hückel-type ion cloud and is 0.88 for DNA and 0.82 for polyphosphate (7). Experimental support for Eq. [4] comes largely from divalent metals, oligopeptides (9), and polyamines (5, 9, 24) where the theory correctly predicts the number of charged groups of the polycation. The standard state of 1 M is used as it is reported that this is approximately the concentration of cations condensed on polyelectrolytes (8); consequently, there is little entropy change for release of these cations near the standard state. Protein–polyelectrolyte binding could be driven in theory by Coulombic forces. The fact that log K_a versus log[salt] plots for many DNA and RNA binding proteins have small y-intercepts, however, supports the view that entropy gain from releasing condensed cations often drives protein, or macroion binding (3). This has also been verified by van't Hoff analysis (5).

Based on Eq. [2] the low slopes observed with most of the potassium salts for PPX–polyphosphate binding suggest few anions or cations are released on polyphosphate binding. With a strong chaotrope such as KSCN it appears 3 $\mathrm{SCN^-}$ bind to PPX and are then released on polyphosphate binding. With PPX able to bind a number of anions suggesting existence of a positively charged binding pocket, one would expect a similar number of condensed potassium cations to be released upon PPX binding to polyphosphate; however, this does not appear to be the case. Counterion release could occur with polyphosphate–PPX binding but go undetected due changes in the activity of macroions with changes in salt concentration. Effects of counterion release could also be obscured by release of a large amount of structured water or an uptake of cations by PPX accompanying polyphosphate binding. However, the linearity of the data between 150 and 350 mM for all but $KOOCH_3$ makes it seem unlikely that bound-water release or changes in the activity coefficient ratio are exactly countering a larger release of cations or anions. Manning's counterion condensation theory may not be adequate for describing polyphosphate-condensed counterions or changes in the ionic double layer following polyphosphate binding to PPX. Nevertheless, the lack of cation-concentration effects and the linearity of the data over a large salt concentration range suggest that something other than the entropy of condensed cation release drives binding.

Anion effects on $\partial \log K_a/\partial \log[\text{salt}]$ have only been observed with two related helix destabilizing, single-strand binding (SSB) proteins. One is gp32 (25) and the other is SSB of *E. coli* (26). As with PPX, the extent of anion effects for these proteins follows the Hofmeister series. However, for these proteins even salts with the smallest effect on the DNA-protein interaction gave relatively large values of $\partial \log K_a/\partial \log[\text{salt}]$, approximately 4 with *E. coli* SSB and 3 with gp32 (25). This suggests that significant cation release occurs for these protein–polyelectrolyte interactions. The polynucleotide–gp32 interaction is more like the PPX–polyphosphate interaction, as the concentration of certain anions seems to have a greater effect on $\partial \log K_a/\partial \log[\text{salt}]$ than the concentration of any cation (25). While chloride is the most chaotropic species used in the gp32 study, even with NaCl it appeared that more Cl^- were released from the protein than Na^+ from the RNA. This clearly indicated that RNA phosphates could not simply replace Cl^- on the protein, as additional sodium cations would have been released. The mechanism of polyphosphate binding to PPX, like that of polyelectrolytes binding to gp32 or *E. coli* SSB, has not yet been resolved. However, the mechanisms of polyelectrolyte binding for these three proteins appear to be different from those of other protein–polyelectrolyte interactions. Molecular models may be required to accurately describe this novel mechanism. Molecular Poisson–Boltzmann and Monte Carlo models have been developed for protein polyelectrolyte interactions, although these have been applied only to anion choice independent interactions (27, 28).

Salt Effects on the Polyphosphate-C-Terminal Domain Interactions

Interestingly, polyphosphate binding data for the C-terminal domain shows anion choice and salt concentration effects similar to those of the PPX–polyphosphate interaction. This data, along with the similarity of K_a values for PPX and the C-terminal domain (2), supports this domain playing a dominant role in polyphosphate binding by PPX. Further, size exclusion chromatography indicates the C-terminal domain is monomeric (data not shown). Although PPX is dimeric, the similarity of polyphosphate binding by PPX and its C-terminal domain supports the polyphosphate interaction being with only one monomer subunit of the enzyme. This would mean PPX dimers have at least two strong C-terminal polyphosphate binding sites. This may allow cross-linking and formation of large aggregates when inactive PPX interacts with polyphosphate, consistent with the observed precipitation of inactive PPX when polyphosphate is added. This cross-linking may not occur with polyphosphate binding to monomeric C-terminal domain which has only one polyphosphate-binding site. In this case, many additional convenient solution phase methods may be used for measuring polyphosphate binding by the C-terminal domain. This would make the C-terminal domain an attractive alternative to native PPX for characterizing polyphosphate binding by PPX.

Conclusion

The effects of salt choice and concentration on polyphosphate binding by PPX are clearly very different than those observed for other known protein–polyelectrolyte interactions. In particular, the cation concentration independence of polyphosphate binding by PPX seems especially unique and suggests polyphosphate binding by PPX is not driven by entropy from release of polyelectrolyte condensed cations. The anion dependence of this interaction is also distinctly different than observed with nearly all DNA and RNA binding proteins or polypeptides. This anion dependence is similar only to that of gp32 from bacteriophage T4 and SSB of *E. coli*. These results suggest PPX may share a novel binding mechanism with single stranded binding proteins. Experimental values of enthalpic and entropic contributions to binding and a molecular model for the PPX–polyphosphate interaction may be necessary for describing the thermodynamics of this interaction.

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